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Synthetic Influenza Viral Double-Stranded RNA Induces an Acute-Phase Response in Rabbits

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Numerous studies have characterized the physiological effects of synthetic, high-molecular-weight, homopolymeric, double-stranded RNA (dsRNA), particularly polyriboinosinic·polyribocytidylic acid [Carter and De Clercq (1974): Science 186:1172–1178], but limited information exists regarding the physiological effects of dsRNA of viral composition and size. In this report, we determined sleep and fever responses of rabbits to intracerebroventricular injection of different doses of synthetic viral dsRNA (either 108 base pairs or 661 base pairs) derived from the N-terminal sequence of gene segment 3 of the A/PR/8/34-H1N1 (PR8) influenza virus. Both the108-mer and the 661-mer dsRNAs increased nonrapid eye movement sleep, suppressed rapid eye movement sleep, and induced fever. The 661-mer dsRNA had more potent somnogenic and pyrogenic effects than the 108-mer dsRNA on the basis of weight. Neither single-stranded RNA from the corresponding sequences had significant effects on sleep or brain temperature. These results demonstrate for the first time that low-molecular-weight, viral dsRNA has the stability in vivo that is required to induce the fever and sleep changes found in natural viral infections, and the hypothesis is supported that virus-associated dsRNA may be responsible for initiating the acute-phase response during viral infections. ***J. Med. Virol. 57: 198–203, 1999.***

KEY WORDS: cytokine; virus; double-stranded RNA; sleep; fever

INTRODUCTION

Many acute viral infections are associated with excess sleep and fever. Previously, we characterized the sleep and body temperature alterations induced by influenza A virus in both rabbits [Kimura-Takeuchi et al., 1992a] and mice [Fang et al., 1995]. It has been proposed that such responses are induced by viral double-stranded RNA (dsRNA) made as a by-product of viral replication [Carter and De Clercq, 1974]. In support of this hypothesis, we have shown that the high-molecular-weight, synthetic dsRNA, polyriboinosinic·polyribocytidylic acid [poly(rI·rC)], induces characteristic sleep and fever responses in the rabbit [Krueger et al., 1988] and that dsRNA extracted from influenza-infected mouse lungs induces a similar acute-phase response [Majde et al., 1991]. Poly(rI·rC) can also substitute for virus in an exposure regimen that suppresses the acute-phase response [Kimura-Takeuchi et al., 1992b]. However, poly(rI·rC) differs substantially from viral dsRNA in terms of both molecular size and base composition. Because size and base composition are important determinants of stability and, thus, activity of dsRNA in vivo [Torrence and De Clercq, 1977; Krueger et al., 1988], it is important to examine responses to dsRNA of viral composition and of a size that could exist in nature. In this report, we characterize the biological activity of synthetic viral dsRNA in our rabbit sleep and fever model to better determine its potential as a trigger of the viral acute-phase response.

MATERIALS AND METHODS

Animals

Adult New Zealand male rabbits (about 5.0 kg at the time of the sleep studies) were used in the experiments. Animals were maintained in an animal facility that was accredited by the American Association for the Accreditation of Laboratory Animal Care. Experimental protocols were approved by the institutional animal care and use committee. The rabbits were anesthetized with ketamine and xylazine (35.0 mg/kg and 5.0 mg/kg, respectively). Four stainless-steel screws were im-

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planted over the frontal and parietal cortices for recording electroencephalograms (EEGs). A 30 k-ohm thermistor was implanted over the parietal cortex for recording brain temperature (T_{brain}). An intracerebroventricular (ICV) guide cannula was implanted into the left lateral ventricle. The position of the guide cannula was verified during implantation by a sudden drop in the pressure of an infusion fluid. A minimum of 10 days of recovery from the surgery were allowed before habituation to the experimental cages.

Preparation of Viral dsRNA

The synthesis of single-stranded RNA (ssRNA) fragments of PR8 PA gene (gene segment 3), consisting of either the 5′ terminal 108 nucleotides (108-mer) or the 5′ terminal 661 nucleotides (661-mer), was done according to standard procedures [Sambrook et al., 1989]. To derive the 661-mer, the original PA clone pT3-PR8, which contains the full 2,233 nucleotides of gene segment 3 (kind gift of Dr. P. Palese, Mt. Sinai School of Medicine, New York, NY), was restricted with the endonucleases Eco RI and Hind III (Boehringer Mannheim, Indianapolis, IN). The roughly 700-base pair (bp) fragment produced was isolated by using a QIAquick Gel Extraction Kit (Quiagen, Inc., Chatworth, CA) according to the manufacturer's directions. This fragment, which contains the 5′-terminal 661 nucleotides of gene segment 3 and part of the vector sequence, was inserted into the vector pGEM-3 (Promega, Madison, WI), which had been linearized by using Eco RI and Hind III, yielding the plasmid pGEM3-H/EPR8. Plasmid pGEM3-H/EPR8 was linearized with Eco RI or Hind III, and RNA in the antisense or sense orientation was prepared by in vitro transcription using 20 units of T7-RNA or SP6-RNA polymerases (Boehringer Mannheim), respectively, in a final volume of 20 μl. The sequence of the final product was determined by standard methods; it matched that of the published sequence [Lamb and Choppin, 1983] of the third genomic segment of A/PR/8/34. The preparation of 108-mer ssRNA [Bredow et al., 1995] was slightly different, in that the first 108 bases of gene segment 3 were synthesized commercially in the sense and antisense orientations. Equimolar amounts of the two DNA oligomers were annealed in Tris/EDTA buffer overnight. An aliquot of the dsDNA was digested with Hind III and Eco RI. Then, the dsDNA was inserted in into the vector pGEM-3 in the same manner as 661-mer fragment.

The dsRNA was obtained by annealing the sense and antisense RNA oligomers. After two precipitations with 3 volumes of absolute ethanol, equal amounts of the two RNA oligomers were annealed at 50°C in a buffer of pH 7.9 containing 10 mM Tris/EDTA, 0.4 M NaCl, 10 mM $MgCl_2$, and 50% formamide. The annealed RNA was then precipitated with 3 volumes of absolute ethanol and taken up in pyrogen-free 0.9% NaCl.

Administration of dsRNA

Synthetic ssRNA and dsRNA fragments of either 108 or 661 bases were examined for their effects on sleep and temperature. Based on our previous results with poly(rI·rC) in the same model [Krueger, et al., 1988], a range of doses was tested, starting with 1,000 ng/rabbit of the 108-mer dsRNA and working down in dose until no response could be detected. Rabbits were injected with different doses of ssRNA (250, 500, or 1,000 ng/rabbit for 108-mer ssRNA; 30 or 100 ng/rabbit for 661-mer ssRNA) on control days and with different doses of dsRNA (100, 250, 500, 1,000, or 2,000 ng/rabbit for 108-mer dsRNA; 30 or 100 ng/rabbit for 661-mer dsRNA in 25 μl) on experimental days through the ICV cannula. Each control or experimental day was preceded by a baseline day on which the animals received ICV injections of 25 μl pyrogen-free saline. Each injection was carried out over a period of about 2 minutes. The injection cannula was left in the ICV guide cannula for an additional 30 seconds. All injections were made between 0900 hours and 1000 hours. After injection, the animals were connected to the recording system, and EEG, T_{brain}, and motor activity were recorded for 6 hours. In some rabbits, rectal temperature (body temperature; T_{body}) was measured during the injection period and at the end of recording.

Sleep Recording

A 12:12 hour light:dark cycle (lights on at 0600 hours) and an ambient temperature of 21 ± 1°C were maintained in the experimental chambers. Animal recordings were carried out as described previously [Kimura-Takeuchi et al., 1992a]. The recording system consisted of a 386 microcomputer, a PC30D analog-to-digital converter (Industrial Computer Source, San Diego, CA), and several types of Grass biological amplifiers (Grass Instruments, Inc., Quincy, MA). EEG signals were amplified and filtered below 0.5 Hz and above 30 Hz at 6 db/octave with a Grass wide-band AC EEG preamplifier. Motor activity signals and T_{brain} signals were amplified with separate amplifiers. The signals were sampled at 128 Hz for EEG, 2 Hz for motor activities, and 2 Hz for T_{brain} through the analog-to-digital converter and were displayed on the monitor. The EEG data were subjected to on-line fast Fourier transformation (FFT). Power density values (μV^2 at 0.5 Hz resolution) were computed for every 2-second periods and were averaged for 10-second periods. All data for EEG, motor activity, and T_{brain} and results of FFT were saved to the hard disk drive.

Sleep-wake states were scored visually after data collection as described previously [Kimura-Takeuchi et al., 1992a]. Sequential 10-second epochs of EEG, motor activity, T_{brain} data, and the results from FFT of EEG signals were displayed graphically on the computer screen. The vigilance states were defined according to the following criteria: non-rapid eye movement sleep (NREMS) was identified by high-voltage delta waves in the EEG, decreased motor activities, and declining T_{brain} at NREMS onset with temperature stabilized after a few minutes; rapid eye movement sleep (REMS) was identified by low-amplitude, fast activity in the EEG, absence of motor activities, and rapid increase of

T_{brain} at REMS onset; and wakefulness was defined by low-voltage EEG activity, increased and varying levels of motor activities, and slow increase of T_{brain} after awakening. After scoring, the EEG power spectral data were sorted further and were averaged hourly according to the behavioral states of the experimental animals. The summed power from 0.5 Hz to 4.0 Hz during NREMS was used as an indicator of EEG slow wave activity (SWA). The power spectrum values were normalized by using the averaged baseline SWA power during NREMS as 100.

Statistical Procedures

Sleep (percentage of time in NREMS and REMS), EEG SWA, and T_{brain} data were analyzed with two-way, repeated-measure analyses of variance (AVOVA). For the analysis of sleep data, the first independent variable was treatment, and the second independent variable was time. If the effect of treatment or the interaction (between treatment and time) was significant, then a Student-Newman-Keuls (SNK) test was used to compare multiple means. In all conditions, the effect was considered statistically significant if $P \leqslant 0.05$.

RESULTS

Effects of the 108-Mer dsRNA and 108-Mer ssRNA

The effects of the 108-mer dsRNA on sleep, T_{brain}, and T_{body} are shown in Figure 1. NREMS increased significantly during the 6-hour recording period by the 108-mer dsRNA at 100 ng [$F_{(1,7)} = 8.687$, $P < 0.025$], 500 ng [$F_{(1,12)} = 26.16$, $P < 0.0025$], 1,000 ng [$F_{(1,7)} = 7.094$, $P < 0.05$], and 2,000 ng [$F_{(1,7)} = 6.12$, $P < 0.05$] doses and showed similar increases after 250 ng ($P = 0.1405$) of dsRNA, but that increase did not achieve statistical significance. The increases in NREMS had a latency about 2 hours. REMS did not changed significantly after 100 ng and 250 ng of 108-mer dsRNA treatment; however, it decreased significantly after 500 ng [$F_{(1,6)} = 22.34$, $P < 0.005$], 1,000 ng [$F_{(1,7)} = 6.61$, $P < 0.05$], and 2,000 ng [$F_{(1,7)} = 6.26$, $P < 0.05$] doses of dsRNA.

T_{body} increased significantly with all doses of dsRNA (Table I). T_{brain} displayed increases similar to those seen in T_{body} after dsRNA treatment (Table I). There was also a latency of about 2 hours from the time of dsRNA injections and the onset of fever. In contrast, there was no significant change in sleep after injections of different doses of either strand of 108-mer ssRNA. The data for both strands of ssRNA were pooled together and are shown in Figure 2. On a molar basis, the doses in Figure 2 match those shown in Figure 1. Neither T_{brain} nor T_{body} was influenced significantly by ssRNA treatment.

Effects of the 661-Mer dsRNA and the 661-Mer ssRNA

The effects of 661-mer dsRNA on sleep, T_{brain}, and T_{body} are shown in Figure 3. NREMS increased significantly by 100 ng of the 661-mer dsRNA [$F_{(1,7)} = 22.21$, $P < 0.0025$]. After the 30-ng dose, NREMS increased slightly, but that increase did not reach statistical significance. REMS was not changed after the 30-ng dose of the 661-mer dsRNA and was significantly suppressed after the 100-ng dose of the 661-mer dsRNA [$F_{(1,7)} = 6.655$, $P = 0.05$]. T_{brain} increased significantly with 100 ng of the 661-mer dsRNA [$F_{(1,7)} = 10.24$, $P = 0.02$]. In contrast, neither strand of ssRNA of the same sequence had any effects on sleep, T_{brain}, or T_{body} at the 100-ng dose.

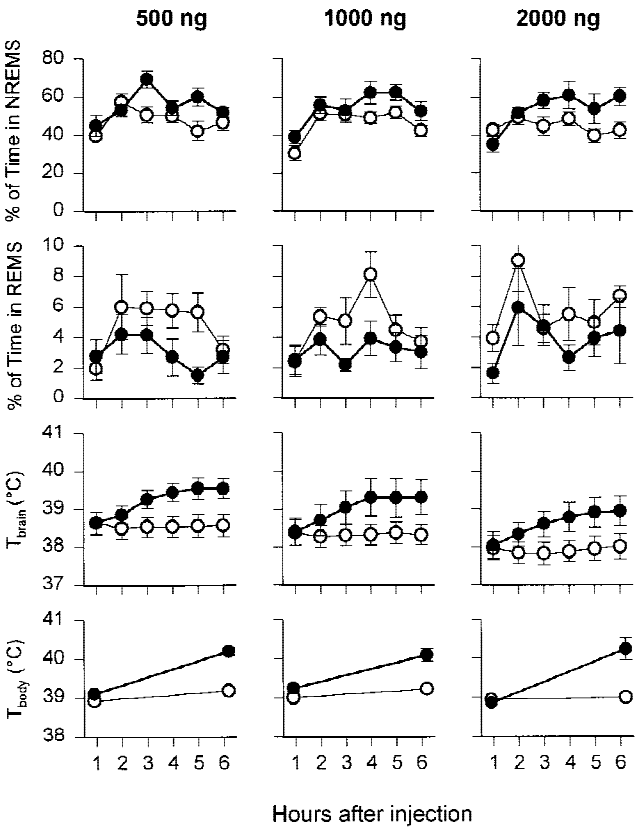

Fig. 1. Effects of the 108-mer viral double-stranded RNA (dsRNA) on sleep, brain temperature (T_{brain}), and rectal temperature (body temperature; T_{body}). Open and solid circles represent data collected after saline or dsRNA injections, respectively. Error bars indicate the standard errors. Nonrapid eye movement sleep (NREMS) increased significantly after different doses of dsRNA, with a latency of about 2 hours. Rapid eye movement sleep (REMS) decreased after dsRNA treatment. For statistical details, see text.

DISCUSSION

These results demonstrate that small fragments of dsRNA of the same base composition as viral RNA are capable of inducing fever and excess SWA sleep when rabbits are inoculated into the cerebral ventricle. These acute-phase responses increase in intensity with increasing concentrations of the synthetic viral dsRNA. A sixfold increase in molecular weight, to a size about one-third that of the average influenza virus gene segment, enhanced only slightly the biological activity of the synthetic viral dsRNA. No sleep or fever responses were seen to the single-stranded oligomers that were used for preparation of the double-stranded oligomers,

TABLE I. Effects of 108-Mer Double-Stranded RNA and 108-Mer Single-Stranded RNA on Brain and Body Temperature*

	Saline		dsRNA		Change		
Doses (ng)	Mean	S.E.	Mean	S.E.	(°C)	F or q values	*P* Values
dsRNA							
T_{brain} (°C)							
100	38.10	0.39	38.60	0.39	+0.50	$F_{(1,5)} = 11.24$	<0.025
250	38.05	0.32	38.74	0.41	+0.69	$F_{(1,6)} = 27.7$	<0.02
500	38.55	0.29	39.22	0.25	+0.67	$F_{(1,5)} = 62.00$	<0.0005
1,000	38.32	0.29	38.97	0.32	+0.65	$F_{(1,7)} = 35.23$	<0.001
2,000	37.91	0.31	38.63	0.35	+0.71	$F_{(1,7)} = 20.60$	<0.005
T_{body} (°C)							
100	39.15	0.05	39.80	0.20	+0.65	$q_{(2,31)} = 5.39$	<0.01
250	39.24	0.09	40.28	0.08	+1.04	$F_{(1,6)} = 34.2$	<0.002
500	39.18	0.08	40.19	0.09	+1.01	$F_{(1,6)} = 76.90$	<0.0001
1,000	39.22	0.05	40.09	0.16	+0.87	$F_{(1,7)} = 41.10$	<0.0005
2,000	39.00	0.06	40.24	0.28	+1.24	$F_{(1,7)} = 17.30$	<0.01
ssRNA							
T_{brain} (°C)							
250	37.89	0.43	37.94	0.49	+0.05	$F_{(1,4)} = 0.26$	n.s.
500	38.00	0.23	38.15	0.23	+0.15	$F_{(1,9)} = 4.44$	n.s.
1,000	37.87	0.35	38.01	0.44	+0.14	$F_{(1,4)} = 3.20$	n.s.
T_{body} (°C)							
250	39.20	0.08	39.30	0.07	+0.10	$F_{(1,4)} = 0.56$	n.s.
500	39.17	0.11	39.23	0.10	+0.06	$F_{(1,9)} = 0.15$	n.s.
1,000	39.08	0.06	39.50	0.18	+0.42	$F_{(1,4)} = 3.04$	n.s.

*dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; T_{brain}, brain temperature; T_{body}, body temperature; S.E., standard error; n.s., not significant.

indicating that the responses to the dsRNA were not due to contamination of the RNA with bacterial endotoxin during preparation [Majde, 1993]. Such contamination is of special concern when bacterial plasmids are employed due to high levels of endotoxin found in these preparations [Wicks et al., 1995].

Our previous studies [Krueger et al., 1988] in the same rabbit model with the high-molecular-weight (>10^6 Da), synthetic dsRNA, poly(rI·rC), demonstrated that 1 ng of this material substantially increased NREMS, decreased REMS, and increased T_{body} and T_{brain} in a manner similar to the oligomers described here; 10 ng was the highest dose of poly(rI·rC) used by the ICV route in this study. Single-stranded poly rI or poly rC were also inactive. A striking finding in this study was that as much as 1,000 ng of high-molecular-weight, double-stranded polyriboadenylic(polyribouridylic acid [poly(rA·rU)] is inert in the rabbit brain [Krueger et al., 1988], pointing to base composition as a more critical parameter than molecular weight in determining the biological response. Early studies with synthetic dsRNA homopolymers revealed that interferon induction correlated with resistance of the homopolymers to serum nucleases, poly(rI·rC) being much more active than poly(rA·rU) [Black et al., 1973]. Nuclease resistance of dsRNA heteropolymers, such as the synthetic viral dsRNA described here, has received limited study, although the presence of (rA·rU) regions in native dsRNA might render these molecules more nuclease-sensitive than poly(rI·rC). However, phenol-extracted picorna virus replication intermediates have higher melting temperatures, are more resistant to serum nuclease activity, and are more potent interferon

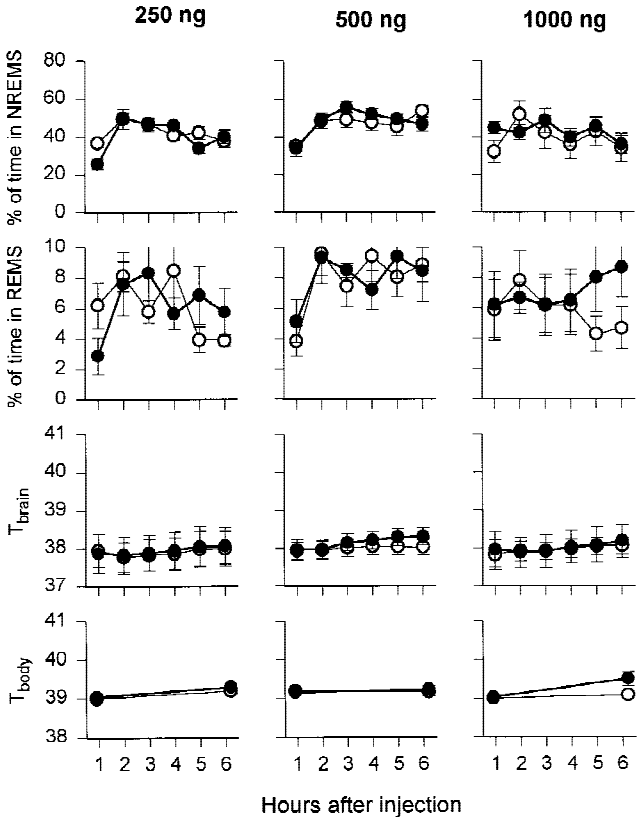

Fig. 2. Effects of the 108-mer viral single-stranded RNA (ssRNA) on sleep, T_{brain}, and T_{body}. Open and solid circles represent data collected after saline or ssRNA (average of both sense and antisense strands) injections, respectively. The doses used (250, 500, and 1,000 ng), on a molar basis, match the doses of 500, 1,000, and 2,000 ng shown in Figure 1, respectively. Error bars indicate the standard errors.

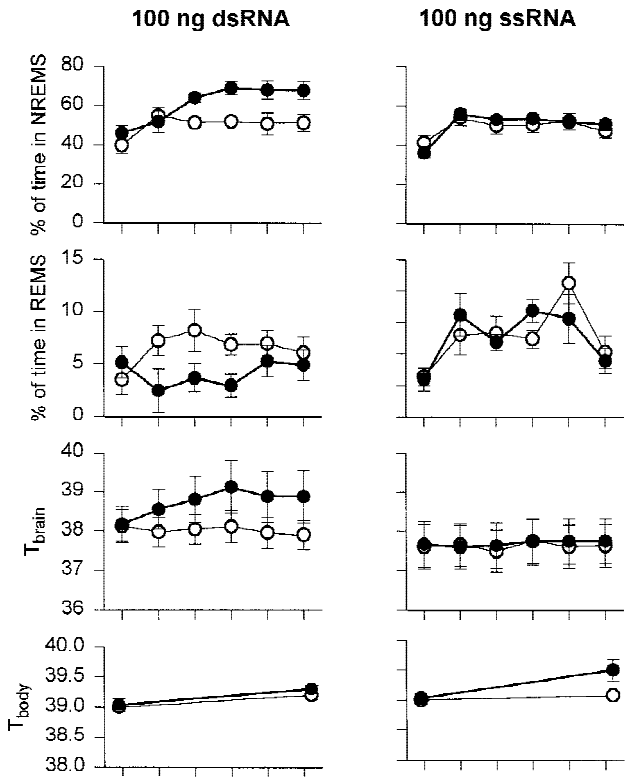

Fig. 3. Effects of the 661-mer viral dsRNA and the corresponding ssRNAs on sleep, T_{brain}, and T_{body}. Open circles represent data collected after saline, and solid circles represent data collected after dsRNA (100 ng/rabbit) or ssRNA (100 ng/rabbit) injections. Injection of dsRNA significantly increased NREMS, suppressed REMS, and significantly increased T_{brain} and T_{body}. Error bars indicate the standard errors.

inducers in vivo than poly(rI·rC) [Falcoff et al., 1973]. In another study in our laboratory [Majde et al., 1998], we have shown that the synthetic viral dsRNA used in this report is highly resistant to nucleases secreted into culture medium by MDCK cells over an 8-hour time span, whereas the ssRNAs that comprise the synthetic viral dsRNA are degraded readily by this culture medium. In this same study, supplementation of the nuclease-rich medium with a concentration of exogenous RNase A, which has been shown to degrade ssRNA but not dsRNA, also failed to degrade the 661-mer dsRNA. Therefore, it appears that heteropolymeric dsRNA of viral composition have nuclease-stability characteristics that are more similar to poly(rG·rC) [Torrence and De Clercq, 1984] than to poly(rA·rU), indicating that the presence of adjacent (rG·rC) base pairs stabilizes (rA·rU) regions of the heteroduplex against nucleolytic attack.

This study and a related, earlier report from our laboratory [Bredow et al., 1995] are the first to demonstrate that small fragments of dsRNA of viral composition can induce an acute-phase response in vivo, presumably through induction of proinflammatory cytokines [Majde et al., 1991]. An early study showed that interferon activity could be induced in rabbits by intravenous challenge with microgram quantities of heteroduplex dsRNA extracted from retrovirus or dsRNA phages [Hilleman, 1970]. We have shown that interferon-α induces a similar acute-phase response in the rabbit [Kimura et al., 1994], as does poly(rI·rC) or the oligomeric dsRNAs described in this report, although we anticipate that other cytokines in addition to interferon-α may mediate the acute-phase response to dsRNA [Majde et al., 1991]. Synthetic, heteroduplex dsRNA that was of intermediate size (309 bp) between the two oligomers used in our study was demonstrated to induce interferon-β and interleukin-1α gene expression in human cells in vitro [Haines et al., 1992]. The 108-mer used in these studies approached the lower molecular size limit of heteroduplex RNA (30 bp) that has been shown to be capable of activating the dsRNA-activated protein kinase R (PKR) [Manche et al., 1992]. Activation of PKR by dsRNA is required for activation of the transcription factor NF-κB by dsRNA [Maran et al., 1994], and activation of NF-κB is likely to play a significant role in the proinflammatory cytokine-induction that is responsible for the acute-phase response observed in this study. However, the lower size limit of heteroduplex RNA that is required for induction of cytokines in vivo has yet to be defined, although our studies indicate that it is probably less than 108 bp. These data suggest that small fragments of viral dsRNA replication intermediates have the stability in vivo that is required to induce an acute-phase response, and they lend support to the hypothesis that viral dsRNA can play a role in viral systemic disease. Evidence now exists that viral dsRNA is released from dying cells [Majde, et al., 1998]; however, further investigations are required to characterize its distribution and mode of action in virally infected tissues and organisms.

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